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Research Article

Application of Bioinformatics and Molecular Dynamics Simulation for Designing FGF7 Mutants Stable in Urea Solution

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Abstract

Background: FGF7 is a potent cytoprotective and regenerative protein on the injured epithelial tissues, indicating its potential therapeutic effect in conditions such as mucositis, ulcerative colitis, and cutaneous wound formation induced by chemotherapy or radiotherapy. However, FGF7 low stability prevents its usability as a pharmaceutical product, necessitating the design and production of stable FGF7 mutants.

Methods: In the current study, bioinformatics and MD simulations approaches were used for identifying potential stable mutants of FGF7. For this, the model structure of FGF7 was constructed and subjected to MAESTROweb service to identify stability conferring mutations. The identified potential mutants of FGF7 were MD simulated in an explicit denaturant condition and their stabilities were evaluated. The complexes of FGF7 mutants with FGFR2 receptor were also examined to investigate the receptor binding ability of proposed mutants.

Results: Investigation of stability parameters showed that introducing A104S mutations in FGF7 may greatly improve its stability in urea solution. Calculation of binding energies indicated that A104S mutant interacts better then wild type FGF7 with the receptor. The structural stability and binding capability of A104S were comparable to that of A120C mutation, as an experimentally determined stability conferring mutation, which was used in whole process to indicate the validity of employed MD simulation for predicting the effect of mutation on FGF7 stability.

Conclusion: The results of current study showed that A104S mutant of FGF7 has a potential to be evaluated further for the purpose of presenting a therapeutic agent effective in conditions such as mucositis, ulcerative colitis, and cutaneous wound formation induced by chemotherapy or radiotherapy.

Introduction

Fibroblast growth factor 7 (FGF7), also known as keratinocyte growth factor (KGF), belongs to the FGF7 subfamily and is a multifunctional mesenchymal-epithelial signaling growth factor.[1,2] FGF7 is primarily produced by cells of mesenchymal origin and strongly activate FGFR2b.[3] This protein was first isolated as an epithelial mitogen from human embryonic lung.[4] Members of the FGF7 subfamily (FGF7, FGF10 and FGF22) are essential for organogenesis and tissue patterning in the embryo, and mediate wound healing and tissue homeostasis in adult mammals.[5-8] In a model of inflammatory bowel disease (IBD), FGF7 knockout mice exhibited more severe colonic inflammation and a delay in tissue repair compared with wild-type mice.[9] Furthermore, evidenced by *in vivo* experiments, FGF7 was over expressed upon injury and inflammatory conditions[10] and presented potent cytoprotective and regenerative effects on the injured epithelial tissues.[11,12] These findings indicate that FGF7 plays a crucial role in epithelial preservation and/or repair processes. Due to FGF7 cytoprotective and healing functions, FGF7 is considered as a potential therapeutic agent in conditions such as mucositis, ulcerative colitis, and cutaneous wound formation induced by chemotherapy or radiotherapy.[13-15] To play its role, FGF7 needs to be stable enough to reach the target cells with proper conformation. However, several studies have underlined the low stability for most FGFs, including the FGF7 subfamily.[16] Polyanions such as heparan sulfate (HS), sucrose octasulfate (SOS), Polyethylene glycol (PEG), and inositol hexaphosphate (IHP) have been frequently used to stabilize the FGFs subfamily.[17,18] Introducing mutations in the structure of proteins by either *in silico* or experimental approaches is a well known method for improving protein stability.[19] To this end, *in silico* methodologies enable identification of potential stability-conferring mutations in a protein

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of interest prior to performing experiments in a cost and time saving manner. Up to now, *in silico* and experimental studies have been carried out to improve the stability features of FGF7.[20-23] Urea and guanidinium hydrochloride (GdmCl) are two chemical denaturing agents which have been frequently used in the study of protein folding and stability in *in silico* studies.[24,25] The current study aimed to use bioinformatics tools to identify mutants of FGF7 with higher conformational stability in an explicit urea-water environment. MAESTROweb enabled full screening of FGF7 sequence to identify the most potential stability enhancing mutations. The identified mutations were further evaluated by molecular dynamics simulations when the behavior of mutants was monitored in urea solution. Finally, the binding capabilities of proposed mutants to FGFR2 were calculated.

Methods

FGF7 model building and validation

The amino acid sequence of human FGF7 without signal peptide was retrieved from the UniProtKB database (https://www.uniprot.org/uniprot/P21781). FGF7 model structure was constructed using I-TASSER from its web server.[26,27] Ramachandran plot, PROCHECK,[28] PROVE,[29] and Verify3D[30] programs were used to evaluate the model.

Identification of stability inducing mutations

A machine learning-based procedure namely multi agent stability prediction upon point mutations (MAESTRO) tool was employed from its web server (MAESTROweb at biwww.che.sbg.ac.at/maestro/web) to identify mutations which may improve the stability of FGF7. To do this, the residues of FGF7 were virtually mutated to all other natural amino acids one at the time using MAESTRO program[31] and the effect of each mutation on the stability of FGF7 was calculated. The residues of FGF7 responsible for binding to FGFR2 and heparan sulfate were excluded from the study. According to the MAESTRO algorithm the mutations with negative stability values ($\Delta\Delta G° < 0$) were considered as the favorable mutations. The predicted stable mutants of FGF7 were generated using DeepView program (version 3.7)[32] for further evaluations.

Molecular dynamics simulations

The thermodynamic and structural stability of FGF7 and its mutants were explored in explicit 8 M urea solution. The Assisted Model Building with Energy Refinement (AMBER) suite of programs (version 14) operating on a Linux-based (Centus 6.8) GPU work station was used for MD simulations.[33] To this end, AMBER input files were generated using the leap module including the Amber99 force-field, followed by neutralizing the system total charge using the correct number of counter ions (Na^+ or Cl^-). The neutralized system was solvated in a pre-equilibrated 8 M urea box available in AMBER 14 and then subjected to a short energy minimization process using Sander module (500 steps of steepest descent followed by 500 steps of conjugate gradient). The temperature of system was increased from 0 to 298 K in NVT ensemble for 50 ps and after a 50-ps density equilibration (for 500 ps), the system was equilibrated for 500 ps at 298 K with a time step of 2 fs at NPT ensemble with 1 bar pressure (controlled with Langvin thermostat). SHAKE algorithm was utilized to constrain the bond lengths involving hydrogen atoms. The final MD simulation was individually carried out using the particle mesh Ewald (PME) method for 100 ns using NPT ensemble to calculate long-range electrostatic interactions. The coordinates were written out every 10 ps to obtain the trajectory of the simulation. All trajectories were analyzed and compared with each other in terms of structural variations expressed by root mean square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface area (SASA), and radius of gyration (Rg). Total energy of the systems for entire 100 ns simulation was used to compare the stability of FGF7 and its mutants from energy point of view.

MD simulations were also used for exploring the binding affinity of FGF7 mutants to FGFR2. For this, the complexes of receptor with FGF7 mutants were generated by superimposing the constructed models of FGF7 mutants on FGF10 in FGF10-FGFR2 crystal structure (PDB ID: 1NUN). Then, MD simulations were performed as described in previous section at 298 K for 50 ns and the trajectories were used for the calculation of ligand-receptor binding energies based on MM-GBSA and MM-PBSA algorithms.

Results and Discussion

Production of medicines with high stability is a crucial step in pharmaceutical industry especially in biopharmaceutical products. FGF7, as an endogenous protein, has the cytoprotective and regenerative effects on the injured epithelial tissues indicating its therapeutic potential for wound healing. However, wild type FGF7 is a low stable protein in physiological conditions, necessitating attempts for designing its more stable and active forms. Palifermin is the pharmaceutical form of FGF7, which has been designed and produced to solve the stability problems regarding FGF7. Palifermin is more stable than the wild type and is being used for the prophylaxis and management of oral mucositis by protecting oral and intestinal epithelia from the effects of radiation and chemotherapy. Although palifermin is relatively stable, production of further stable and active FGF7 mutants seems a necessity for pharmaceutical industry. In this regard, some experimental and *in silico* studies have been carried out.[20-23] An *et al.*[21] improved FGF7 thermal stability by introduction of mutations in close vicinity of disulfide bond and surface salt bridge. They found that A120C, K126E, and K178E mutations could increase the thermal stability of FGF7 by induction of disulfide bond and ionic interactions. While Kalhor *et al.*[23] used multiple sequence alignment followed by MD simulations for identifying and evaluating stability inducing mutations. In the current

study, MAESTROweb was used for identifying stability conferring mutations. The identified mutations were evaluated by MD simulations which were performed in an explicit urea-water environment. Urea and guanidinium hydrochloride (GdmCl) are used frequently as chemical denaturing agents for studying protein folding and stability in both experimental and in silico studies.[24,25] Urea disrupts hydrogen bonds between water molecules to let them penetrate in deeper hydrophobic sites of proteins, facilitating protein unfolding process.[34] The proposed mechanism indicates that the mechanism of protein unfolding in pure water and urea-water solution is the same.

FGF7 model building and validation

The FGF7 model was constructed using I-TASSER program. I-TASSER uses LOMETS algorithm to select top ten templates with the highest significance (measured by the Z-score) in the threading alignment.[35] FGF10-FGFR2b (PDB ID 1NUN) was the most selected template by multiple threading programs implemented in the LOMETS program. I-TASSER automatically used these templates to generate a large ensemble of structural conformations. Using SPICKER program implemented in I-TASSER,[36] the first model was selected whose C-score was the highest (-2.27) calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. The TM-align value, which is derived from the structural alignment of selected model to all structures in the PDB library, was 0.703 for FGF10-FGFR2b crystal structure, indicating the validity of the constructed model. Evaluation of FGF7 model using VERIFY3D program showed that 83.44% of residues (more than 80%) in the model have averaged 3D-1D score >= 0.2. PROCHECK analysis revealed that based on Psi-Phi degrees, 98.6% of residues are in allowed regions. Based on PROVE calculations, average Z-score of all atoms is 0.043 which is between -0.10 and 0.10, indicating the high quality of built model.

Identification of stability inducing mutations

To predict the effects of different point mutations on the stability of FGF7 3D structure, MAESTROweb was employed which differs this study from the other *in silico* based methodologies.[22,23] MAESTROweb is an easy to use structure-based web service which allows the prediction of changes in stability for user-defined mutations and provides scan functionality for the most (de)stabilizing n-point mutations. In the current study, the model structure for FGF7 was submitted to MAESTROweb and the stability changes upon point mutations were explored based on the evolutionary algorithm at pH 7, when maximum point mutation was set to 1. The mutations related to the FGF7 residues responsible in receptor and heparan sulfate binding as well as the mutations concerning to the conserved residues in FGF family were excluded from evaluation (Figure 1). According to the MAESTRO algorithm the mutations with negative stability values ($\Delta G° < 0$) are considered as favorable mutations. The process led to identification of R78C ($\Delta\Delta G°$= -0.54), A104S ($\Delta\Delta G°$= -0.42), N138I ($\Delta\Delta G°$= -0.48), and K140W ($\Delta\Delta G°$= -0.25) as stability inducing mutations. These mutations were totally different from those identified by Kalhor *et al.*[23] who used multiple sequence alignment for screening. We believe that structure-based web services, like MAESTROweb, can be superior to sequence-based approaches for prediction of changes in stability upon point mutations.

Evaluation of stability of identified mutations using MD simulations

In the current study, MAESTROweb was used to recognize single point mutations which can improve the stability of FGF7 Further evaluations were carried out using MD simulations to explore the dynamic behavior of proteins in urea solution.[37-40] To this end, the predicted stable mutants of FGF7 were generated and evaluated in urea solution during 100 ns MD simulations. In experimental studies, A120C mutation on FGF7 has led to an increased stability,[21] therefore, this mutation has been used as the control in all calculations.

Interpretation of RMSD and RMSF values

Structural stability calculations were carried out by studying the behavior of FGF7 and its mutants in urea solution. For this, the molecules were solvated in a pre-equlibrated 8 M urea environment and simulated for 100 ns at 298 K. In RMSD *vs.* time plots, R78C, A104S, and N138I mutant have reached a plateau during simulations and remained constant in the rest of simulation time, while K140W and especially wild type FGF7 never reached a plateau and fluctuated during 100 ns simulations (Figure 2a). Probability distribution calculation showed that most of the conformers of A104S have been populated at RMSD around 4.8 Å (Figure 2b) indicating the least conformational changes for A104S in 8 M explicit urea environment compared to the other mutants and wild type. As seen in Figure 2b, wild type, R78C, N138I, and K140W have resulted in several peaks in probability distribution plot indicating their instability in 8 M urea solution. According to Figure 2, the RMSD *vs.* time plot and probability distribution calculation for A104S mutant are comparable with that of experimentally determined stable, indicating the stability conferring effect of A104S on FGF7. The high stability of A120C mutant during simulations also indicates the validity of employed MD simulations in the current study. Figure 3 shows the root-mean-square fluctuation (RMSF) plot for FGF7 wild type and its mutants during 100 ns MD simulation in urea environment. As seen, R78C, A104S, and N138I presented lower fluctuation compared to wild type FGF7 and K140W, indicating the stability conferring effect of these mutations. Meanwhile, A120C mutation, as a control, resulted in the least residue fluctuations in the FGF7 structure. Close inspection revealed that along with the overall lowered

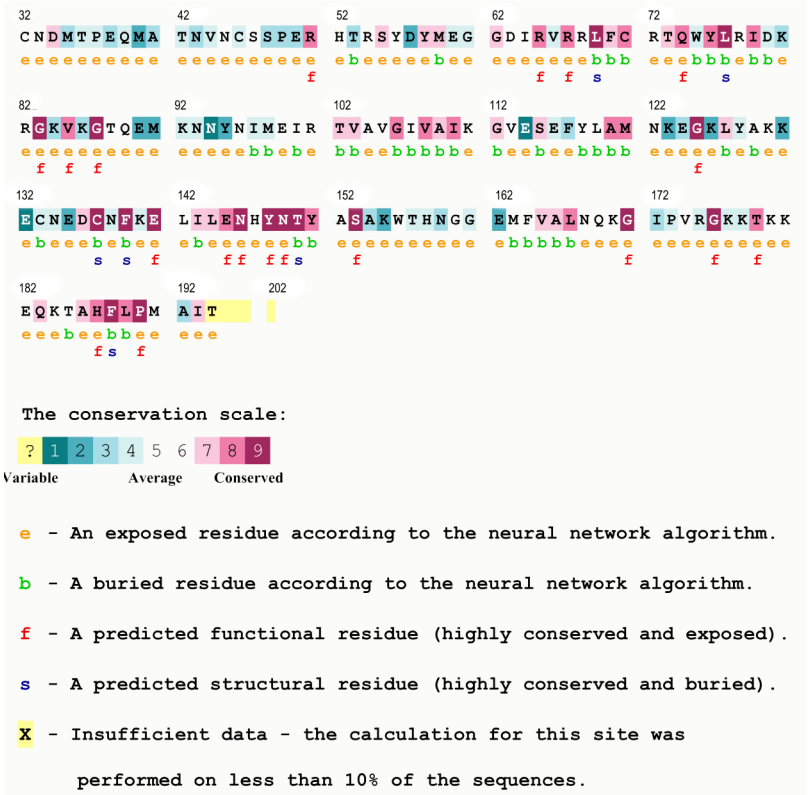

Figure 1. The color-coded sequence of FGF7 colored with ConSurf conservation scores generated by Chimera. The b and e letters below the sequence indicate buried/exposed status of each residue in the 3D model structure of FGF7.

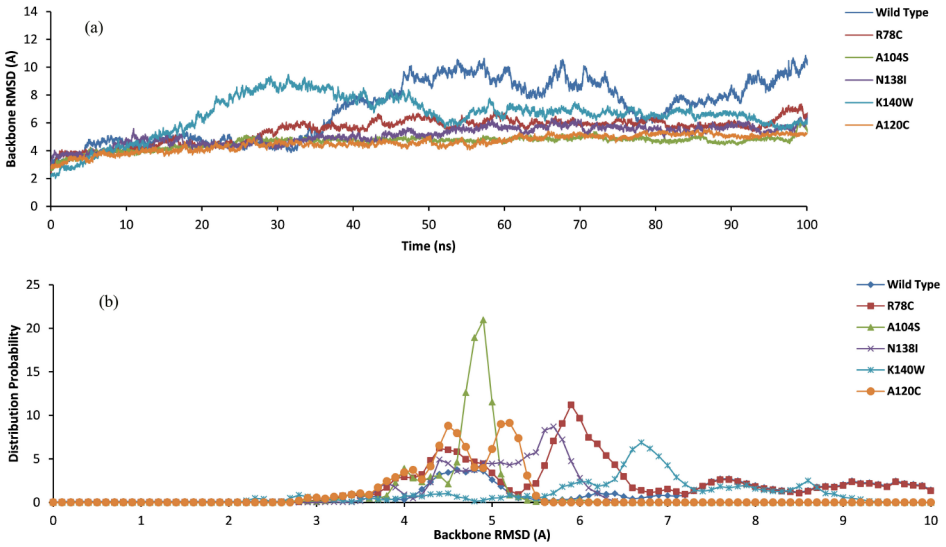

Figure 2. (a) The RMSD alterations for FGF7 and its mutants during 100 ns MD simulations in the urea solution. (b) The distribution probability *vs* RMSD plot for the identified stable mutants.

fluctuations, receptor and heparan sulfate binding residues of R78C, A104S, and N138I mutants are very stable than that of wild type during simulation. These values were the least for the A120C mutant (Figure 3). Binding of FGF7 to heparin sulfate supports its stability[41] and also its dimerization, receptor binding ability, and activity.[42] The results of current work indicate that the identified mutants not only directly improve the stability of FGF7, but also stabilize the heparin sulfate binding domains which can be reflected in the FGF7 activity (Figure 3b).

Solvent accessible surface area

In globular proteins, non-polar amino acids establish hydrophobic interactions to protect the non-polar amino acids in hydrophobic cores from the aqueous environment ensuring the conformational stability of protein. During protein unfolding, the hydrophobic interactions among non-polar amino acid clusters are lost and the hydrophobic

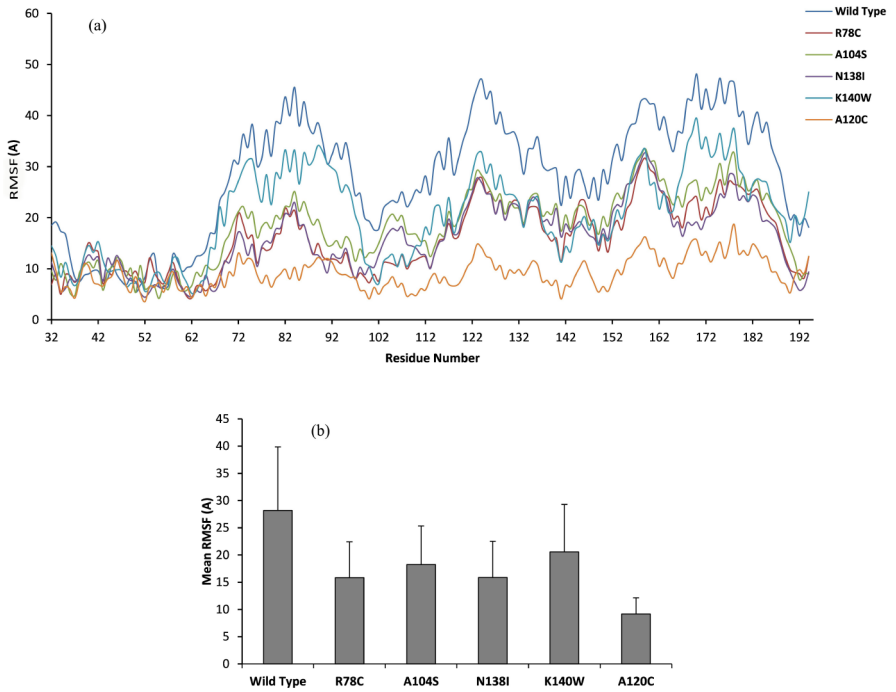

Figure 3. (a) The RMSF alterations and (b) the average RMSF for FGF7 and its mutants during 100 ns MD simulations in the urea solution.

core is exposed to the aqueous surrounding. Therefore, by computing solvent accessible surface area (SASA) the changes in the accessibility of protein to a solvent and hence, the protein resistance to unfolding can be determined. In the current study, SASA of FGF7 variants were calculated using the cpptraj module implemented in Amber package. During the simulations, the hydration of hydrophobic core of wild type, R78C, N138I, and K140W leads to the increased SASA of the FGF7 variants, causing the interruption of hydrophobic interactions among non-polar residues and hence protein unfolding. However, A104S mutant resists against hydrophobic solvation and protein unfolding during 100 ns simulations (Figure 4a). The distribution curves related to the SASA of entire proteins clearly portray the stability of A104S and N138I in explicit urea solution compared to the other variants revealing that the hydrophobic core is more protected from the external environment in A104S and N138I mutants as that of wild type. While K140W and R78C represented larger peak values compared to that of wild type indicating that upon introducing these mutations to FGF7, the hydrophobic core of protein is more likely to be hydrated and accessible to the aqueous environment (Figure 4b). Compared to the experimentally determined stable A120C, A104S represented higher stability to unfolding, indicating that A104S mutant can be considered a potential stable FGF7 variant.

Radius of gyration (Rg)

The Rg of a protein is defined as the distribution of atoms of a protein around its axis. The Rg values of unfolded proteins are typically 1.5 to 2.5 times larger than the radius of gyration of the same proteins in their native globular states.[43] Therefore, calculation of this parameter can be the indicative of protein resistance against unfolding. To this end, in the current study, the Rg values of FGF7 and its mutants were calculated and the results are available in Figure 5. Studies have shown that thermodynamically stable intermediates may exist during the unfolding process.[44] As shown in Figure 5b, wild type FGF7, R78C, N138I, and K140W resulted in several peaks in probability distribution graph, which can be related to the formation of various thermodynamically stable intermediates during the unfolding process, indicating their tendencies to unfolding. While, A104S and A120C have shown the least Rg fluctuations during 100 ns simulations and their probability distribution graphs indicate that A104S mutant is least likely to produce several intermediates, even less than A120C as the experimentally determined stable mutant of FGF7. These analyses support the potential of A104S as the FGF7 stability conferring mutation.

Evaluation of FGF7 mutants on FGFR2 binding

Molecular dynamics simulations can be used to investigate ligand-receptor interaction energies in a dynamic manner in an explicit environment.[45,46] As only A104S and R78C presented stability conferring effects on FGF7, it was decided to perform MD simulations on the complexes of FGFR2 receptor with these mutants and compare the results with that of FGF7 and A120C as the controls. The results of calculations using MM-GBSA and MM-PBSA algorithms on the trajectories produced during 50 ns MD simulations indicated that, R78C and A104S could increase the binding ability of FGF7 to FGFR2 receptor. As shown in Table 1, the binding energy in FGF7-FGFR2 complex was increased from -95.60 kcal.mol^{-1} to -110.43

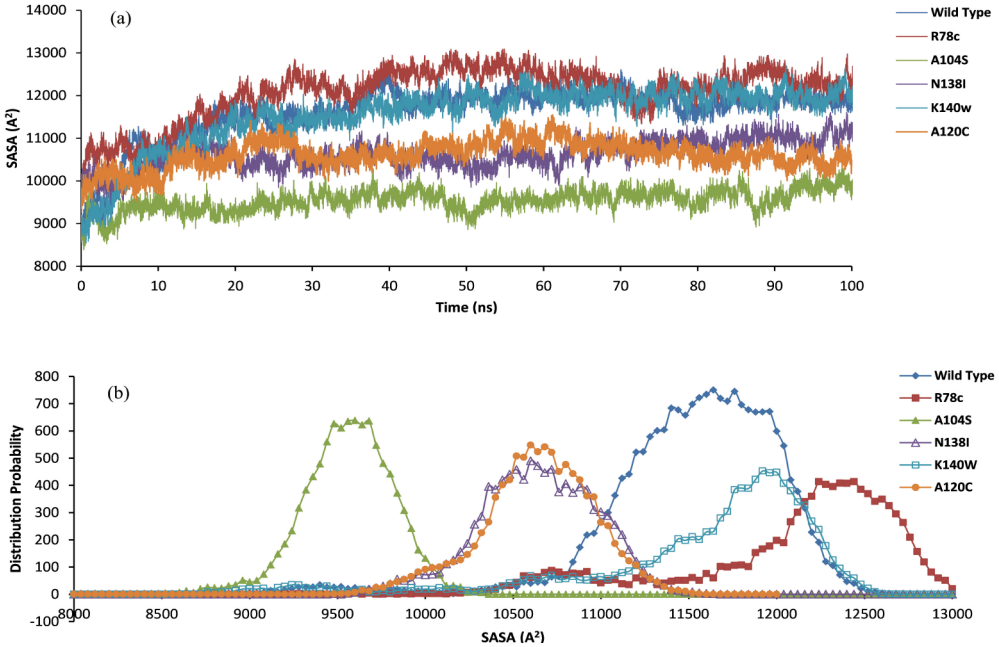

Figure 4. (a) The SASA alterations for FGF7 and its mutants during 100 ns MD simulations in the urea solution. (b) The distribution probability *vs* SASA plot for the identified stable mutants.

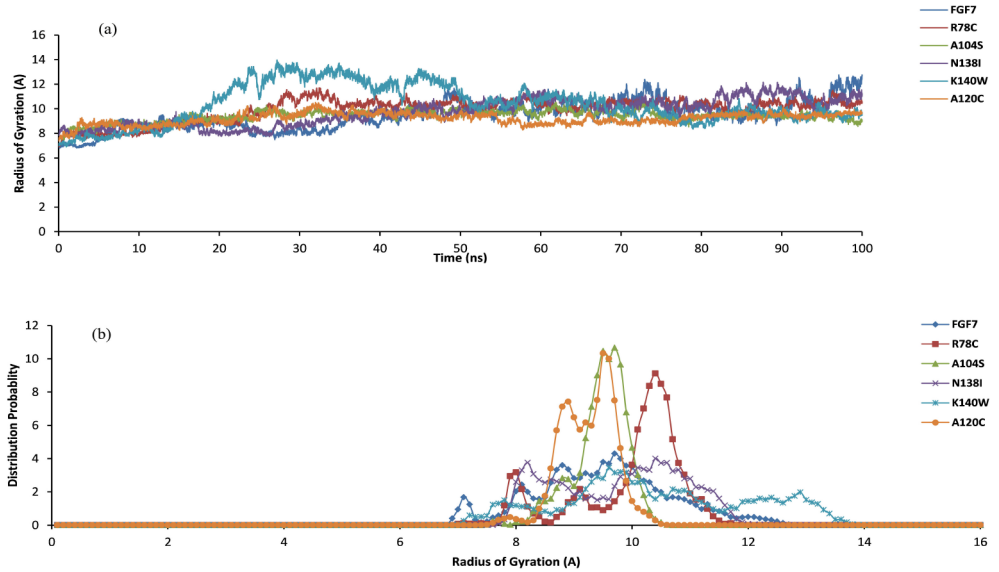

Figure 5. (a) The SASA alterations for FGF7 and its mutants during 100 ns MD simulations in the urea solution. (b) The distribution probability *vs* SASA plot for the identified stable mutants.

and -129.24 kcal.mol^{-1} in A104S and R78C mutants using MM-GBSA algorithm, respectively. In accordance with the experimental data,[21] A120C mutation did not interfere with the binding of FGF7 to FGFR2 ($\Delta\Delta G$ = -104.46 kcal.mol^{-1}) indicating that the MD simulation employed in the current study is able to predict the effects of introduced mutations on FGF7 stability. Calculation of FGF7 binding ability to the receptor using MM-PBSA also revealed the positive role of identified mutations on FGF7 receptor binding (Table 1). These values indicate the preserved and even increased receptor binding ability of identified stable mutants of FGF7.

Conclusion

FGF7 presents potent cytoprotective and regenerative effects on the injured epithelial tissues indicating that FGF7 plays a crucial role in epithelial preservation and/or repair processes. However, due to its low stability, attempts are required to produce stable FGF7 mutants with potential of pharmaceutical use. To this end, bioinformatics and MD simulations approaches were used for identifying potential stable mutants of FGF7. The results showed that introducing A104S mutation in FGF7 may greatly improve its stability to explicit denaturant environment, without affecting its ability to bind heparan sulfate and FGFR2 receptor. The

Table 1. Binding energy calculation of FGF7 and its mutants towards FGFR.2 in 50 ns MD simulations. The binding energies were calculated using MM-GBSA and MM-PBSA algorithms and expressed as kcal.mol^{-1}.

Complex	$\Delta G_{binding}$		SEM	
	GBSA	PBSA	GBSA	PBSA
FGF7-FGFR2	-95.60	-77.48	1.83	2.01
R78C-FGFR2	-129.24	-120.46	1.56	2.08
A104S-FGFR2	-110.43	-102.08	1.58	1.80
A120C-FGFR2	-104.46	-90.99	2.11	2.04

SEM: standard error of mean

identified A104S mutant of FGF7 in the current study is needed to be evaluated by various experimental studies prior to presenting as FGF7 stable mutant.

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Author Contributions

Siavoush Dastmalchi: Conceptualization, Formal Analysis, Writing - Original Draft. Ali Akbar Alizadeh: Conceptualization, Investigation, Formal Analysis, Writing - Review & Editing.

Conflict of Interest

The authors report no conflicts of interest.

References

1. Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. Growth Factors. 2006;24(4):260-7. doi:10.1080/08977190601060990
2. Zinkle A, Mohammadi M. Structural biology of the FGF7 subfamily. Front Genet. 2019;10:102. doi:10.3389/fgene.2019.00102
3. Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem. 2006;281(23):15694-700. doi:10.1074/jbc.M601252200
4. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci U S A 1989;86(3):802-6. doi:10.1073/pnas.86.3.802
5. Beenken A, Mohammadi M. The fgf family: Biology, pathophysiology and therapy. Nat Rev Drug Discov. 2009;8(3):235-53. doi:10.1038/nrd2792
6. Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S, et al. FGF10 acts as a major ligand for fgf receptor 2 iiib in mouse multi-organ development. Biochem Biophys Res Commun. 2000;277(3):643-9. doi:10.1006/bbrc.2000.3721
7. Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, et al. Fgf10 is essential for limb and lung formation. Nat Genet. 1999;21(1):138-41. doi:10.1038/5096
8. Guo L, Degenstein L, Fuchs E. Keratinocyte growth factor is required for hair development but not for wound healing. Genes Dev. 1996;10(2):165-75. doi:10.1101/gad.10.2.165
9. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A 2002;99(22):14338-43. doi:10.1073/pnas.212290499
10. Finch PW, Mark Cross LJ, McAuley DF, Farrell CL. Palifermin for the protection and regeneration of epithelial tissues following injury: New findings in basic research and pre-clinical models. J Cell Mol Med. 2013;17(9):1065-87. doi:10.1111/jcmm.12091
11. Gragnani A, Rossi MB, Albuquerque JC, Brito GS, Aloise AC, Ferreira LM. Keratinocyte growth factor protected cultured human keratinocytes exposed to oxidative stress. Acta Cir Bras. 2010;25(1):93-7. doi:10.1590/s0102-86502010000100019
12. Teranishi S, Kimura K, Kawamoto K, Nishida T. Protection of human corneal epithelial cells from hypoxia-induced disruption of barrier function by keratinocyte growth factor. Invest Ophthalmol Vis Sci. 2008;49(6):2432-7. doi:10.1167/iovs.07-1464
13. Han DS, Li F, Holt L, Connolly K, Hubert M, Miceli R, et al. Keratinocyte growth factor-2 (fgf-10) promotes healing of experimental small intestinal ulceration in rats. Am J Physiol Gastrointest Liver Physiol. 2000;279(5):G1011-22. doi:10.1152/ajpgi.2000.279.5.G1011
14. Sung C, Parry TJ, Riccobene TA, Mahoney A, Roschke V, Murray J, et al. Pharmacologic and pharmacokinetic profile of repifermin (KGF-2) in monkeys and comparative pharmacokinetics in humans. AAPS PharmSci. 2002;4(2):E8. doi:10.1208/ps040208
15. Sandborn WJ, Sands BE, Wolf DC, Valentine JF, Safdi M, Katz S, et al. Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: A randomized, double-blind, placebo-controlled, dose-escalation trial. Aliment Pharmacol Ther. 2003;17(11):1355-64. doi:10.1046/j.1365-2036.2003.01589.x
16. Buchtova M, Chaloupkova R, Zakrzewska M, Vesela I, Cela P, Barathova J, et al. Instability restricts signaling of multiple fibroblast growth factors. Cell Mol Life Sci. 2015;72(12):2445-59. doi:10.1007/s00018-015-1856-8
17. Derrick T, Grillo AO, Vitharana SN, Jones L, Rexroad J, Shah A, et al. Effect of polyanions on the structure and stability of repifermin (keratinocyte growth factor-2). J Pharm Sci. 2007;96(4):761-76. doi: 10.1002/jps.20797
18. Khondee S, Olsen CM, Zeng Y, Middaugh CR, Berkland C. Noncovalent pegylation by polyanion

complexation as a means to stabilize keratinocyte growth factor-2 (KGF-2). Biomacromolecules. 2011;12(11):3880-94. doi:10.1021/bm2007967
19. Alizadeh AA, Jafari B, Dastmalchi S. Application of bioinformatics and molecular dynamics simulation approaches for identification of fibroblast growth factor 10 analogues with potentially improved thermostability. Growth Factors. 2020;38(3-4):197-209. doi:10.1080/08977194.2021.1881501
20. Hsu E, Osslund T, Nybo R, Chen BL, Kenney WC, Morris CF, et al. Enhanced stability of recombinant keratinocyte growth factor by mutagenesis. Protein Eng Des Sel. 2006;19(4):147-53. doi:10.1093/protein/gzj013
21. An YJ, Lee KW, Jung Y-E, Jeong YE, Kim S-J, Woo J, et al. Improvement of fgf7 thermal stability by introduction of mutations in close vicinity to disulfide bond and surface salt bridge. Int J Pept Res Ther. 2022;28(3):85. doi:10.1007/s10989-022-10394-1
22. Poorebrahim M, Sadeghi S, Ghorbani R, Asghari M, Abazari MF, Kalhor H, et al. In silico enhancement of the stability and activity of keratinocyte growth factor. J Theor Biol. 2017;418:111-21. doi:10.1016/j.jtbi.2017.01.009
23. Kalhor H, Sadeghi S, Marashiyan M, Enssi M, Kalhor R, Ganji M, et al. In silico mutagenesis in recombinant human keratinocyte growth factor: Improvement of stability and activity in addition to decrement immunogenicity. J Mol Graph Model. 2020;97:107551. doi:10.1016/j.jmgm.2020.107551
24. Zhang Z, Zhu Y, Shi Y. Molecular dynamics simulations of urea and thermal-induced denaturation of s-peptide analogue. Biophys Chem. 2001;89(2-3):145-62. doi:10.1016/s0301-4622(00)00227-1
25. Zhang D, Lazim R. Application of conventional molecular dynamics simulation in evaluating the stability of apomyoglobin in urea solution. Sci Rep. 2017;7(1):44651. doi:10.1038/srep44651
26. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 2008;9:40. doi:10.1186/1471-2105-9-40
27. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER suite: Protein structure and function prediction. Nat Methods. 2015;12(1):7-8. doi:10.1038/nmeth.3213
28. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. Procheck: A program to check the stereochemical quality of protein structures. J Appl Crystallogr. 1993;26(2):283-91. doi:10.1107/S0021889892009944
29. Pontius J, Richelle J, Wodak S. Deviations from standard atomic volumes as a quality measure for protein crystal structures. J Mol Biol. 1996;264:121-36. doi:10.1006/jmbi.1996.0628
30. Eisenberg D, Luthy R, Bowie JU. VERIFY3D: Assessment of protein models with three-dimensional profiles. Methods Enzymol. 1997;277:396-404. doi:10.1016/s0076-6879(97)77022-8
31. Laimer J, Hiebl-Flach J, Lengauer D, Lackner P. Maestroweb: A web server for structure-based protein stability prediction. Bioinformatics. 2016;32(9):1414-6. doi:10.1093/bioinformatics/btv769
32. Guex N, Peitsch MC. Swiss-model and the swiss-pdbviewer: An environment for comparative protein modeling. Electrophoresis. 1997;18(15):2714-23. doi:10.1002/elps.1150181505
33. Case D, Cheatham T, Darden T, Gohlke H, Luo R, Merz K, et al. The amber biomolecular simulation programs. J Comput Chem. 2005;26:1668-88. doi:10.1002/jcc.20290
34. Bennion BJ, Daggett V. The molecular basis for the chemical denaturation of proteins by urea. Proc Natl Acad Sci U S A. 2003;100(9):5142-7. doi:10.1073/pnas.0930122100
35. Wu S, Zhang Y. Lomets: A local meta-threading-server for protein structure prediction. Nucleic Acids Res. 2007;35(10):3375-82. doi:10.1093/nar/gkm251
36. Zhang Y, Skolnick J. Spicker: A clustering approach to identify near-native protein folds. J Comput Chem. 2004;25(6):865-71. doi:10.1002/jcc.20011
37. Doss CGP, Rajith B, Garwasis N, Mathew PR, Raju AS, Apoorva K, et al. Screening of mutations affecting protein stability and dynamics of fgfr1—a simulation analysis. Appl Transl Genom. 2012;1:37-43. doi:10.1016/j.atg.2012.06.002
38. Li Q, Yan Y, Liu X, Zhang Z, Tian J, Wu N. Enhancing thermostability of a psychrophilic alpha-amylase by the structural energy optimization in the trajectories of molecular dynamics simulations. Int J Biol Macromol. 2020;142:624-633. doi:10.1016/j.ijbiomac.2019.10.004
39. Parveen T, Kamran M, Fatmi MQ. Structural and dynamical thermostability of psychrophilic enzyme at various temperatures: Molecular dynamics simulations of tryptophan synthase. Arch Biochem Biophys. 2019;663:297-305. doi:10.1016/j.abb.2019.01.022
40. Rathi E, Kumar A, Kini SG. Molecular dynamics guided insight, binding free energy calculations and pharmacophore-based virtual screening for the identification of potential vegfr2 inhibitors. J Recept Signal Transduct Res. 2019;39(5-6):415-433. doi:10.1080/10799893.2019.1690509
41. Li X, Wang C, Xiao J, McKeehan WL, Wang F. Fibroblast growth factors, old kids on the new block. Semin Cell Dev Biol. 2016;53:155-67. doi:10.1016/j.semcdb.2015.12.014
42. Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, et al. Heparin-induced oligomerization of fgf molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell. 1994;79(6):1015-24. doi:10.1016/0092-8674(94)90032-9
43. Lobanov MY, Bogatyreva NS, Galzitskaya OV. Radius of gyration as an indicator of protein structure compactness. Mol Biol. 2008;42(4):623-8. doi:10.1134/S0026893308040195

44. Uversky VN, Ptitsyn OB. Further evidence on the equilibrium "pre-molten globule state": Four-state guanidinium chloride-induced unfolding of carbonic anhydrase b at low temperature. J Mol Biol. 1996;255(1):215-28. doi:10.1006/jmbi.1996.0018
45. Alizadeh AA, Dastmalchi S. Designing novel teduglutide analogues with improved binding affinity: An in silico peptide engineering approach. Curr Comput Aided Drug Des. 2021;17(2):225-234. doi:10.2174/1573409916666200217091456
46. Alizadeh AA, Jafari B, Dastmalchi S. Alignment independent 3d-qsar studies and molecular dynamics simulations for the identification of potent and selective s1p1 receptor agonists. J Mol Graph Model. 2020;94:107459. doi:10.1016/j.jmgm.2019.107459